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Letter

De novo transgenerational inheritance of male rat hyperactivity by rotenone

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ABSTRACT — There is growing evidence of transgenerational effects of a single exposure to chemicals, whose mechanism is implicated to be epigenetic. However, it is largely unknown whether psychiatric diseases such as ADHD or autism caused by environmental chemicals might be transmitted. Rotenone (3 mg/kg), a dopaminergic toxin was orally exposed to Wistar male pups at 5-day old. Their spontaneous motor activity was higher 1.3 fold than that of control rats at 11 weeks of age. At 26 weeks of age, the hyperactive rat (F0) was mated with Wistar female rats. We established the two strains of such mating and found the spontaneous motor activity of the offspring (F1) were much higher 1.5~2.0 fold than those of both control offspring and the parents. Thus, in this study I show the rat hyperactivity caused by neonatal rotenone lesions was transmitted to next generation, indicating the *de novo* inheritance.

Key words: ADHD, Rat hyperactivity, Transgeneration, Epigenetics, Rotenone

INTRODUCTION

Attention deficit hyperactivity disorder (ADHD) is characterized by behavioral and cognitive symptoms such as hyperactivity, inattention, disorganization, and impulsivity (Goldman *et al*., 1998; Gozal and Molfese, 2005; Gordon and Mitchell 2009; Rappley, 2005). The etiology is considered to be multifactorial. Interaction of genetic and environmental risk factors would be affected in expression of the disorder. Genetic studies have shown the association of 7-repeat alleles of D4 receptor gene with the occurrence of ADHD (Franke *et al*., 2012; LaHoste *et al*., 1996). Many environmental factors seem to cluster around pregnancy and birth, including maternal smoking, alcohol consumption and stress during pregnancy.

The animal model for hyperactivity disorders was produced by Shaywitz *et al*. (1976), who demonstrated that rat pups treated with 6-hydroxydopamine at 5 days of age developed increased motor activity, leading to cognitive difficulties in shuttle-box learning between 2–4 weeks of age. Following their protocol, we have also demonstrated rat hyperactivity by administration of endocrine disrupting chemicals, such as bisphenol A (Ishido *et al*., 2004a, 2007, 2011), *p*-octhylphenol (Masuo *et al*., 2004), nonylphenol (Masuo *et al*., 2004), dibutylphthalate (DBP; Ishido *et al*., 2005), dicyclohexylphthalate (DCHP; Ishido *et al*., 2004b), diethylhexylphthalate (DEHP; Masuo *et al*., 2004), and *p*-nitrotoluene (Ishido *et al*., 2004c; Ishido and Usu, 2017a). The results of our animal experiments have been supported by many other epidemiological studies (Kim *et al*., 2009; Cho *et al*., 2010; Yolton *et al*., 2011; Harley *et al*., 2013; Chopra *et al*., 2014; Park *et al*., 2015; Huang *et al*., 2015; Philippat *et al*., 2017; Engel *et al*., 2018; Ku *et al*., 2020).

It was shown that vinclozolin, an endocrine disruptor exerted the effects on male fertility and that the effects were transmitted to F_4 generation even only when the original gestating mother (F_0) of F_1 generation received

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a transient chemical treatment (Anway *et al*., 2005). It might be mediated through epigenetic phenomena.

Here, their report allowed us to examine if rat hyperactivity caused by environmental chemical, rotenone toxicity would be transgenerated.

MATERIALS AND METHODS

Materials

Rotenone was purchased from Sigma-Aldrich (Tokyo, Japan). Olive oil was from nakarai tesque Corp. (Kyoto, Japan).

Animals and treatments with chemicals

All animal experiments were carried out in strict accordance with the Experiment and Related Activities in Academic Research Institutions guidelines, under jurisdiction of the Ministry of Education, Culture, Sports, Science and Technology, Japan. The protocol was approved by the Committee on the Ethics of Animal Experiments of the National Institute for Environmental Studies (NIES), Japan. In addition, this study was carried out in compliance with the ARRIVE guidelines. Pregnant Wistar rats were obtained from Clea Japan (Tokyo, Japan). They were maintained in home cages and fed with a standard laboratory chow (MF diet, Oriental Yeast Corp., Tokyo, Japan) and distilled water ad libitum at 22°C on a light-dark cycle (12 hr/12 hr) for at least one week. All animal care procedures were in accordance with NIES guidelines. About 50 male pups were born from 10 pregnant rats and 5–7 pups were randomly housed. The male pups were selected to be 10~14 g body weight at 5 days of age. They were weaned at 3 weeks of age.

Rotenone was suspended in 30% (w/w) milk-oil solution which was composed of nonfat milk (Meiji Co., Tokyo, Japan) and olive oil, and 3 mg/kg of rotenone was orally administered into the pups at 5 days of age. Control rats were administered with vehicle (30 μL) alone.

Mating

Control or the treated male rats (F_0) were mated with females from different litters, respectively at 26~30 weeks of age (Fig. 1). The offspring (F_1) were obtained, and their spontaneous motor activity was measured by Supermex system as below. Two F1 strains were established, and designated as epi5A and epi5B, respectively.

Measurements of spontaneous motor activity

To examine the behavioral effects of rotenone, we employed the Supermex system (Muromachi Kikai, Tokyo, Japan). A Supermex sensor head consists of

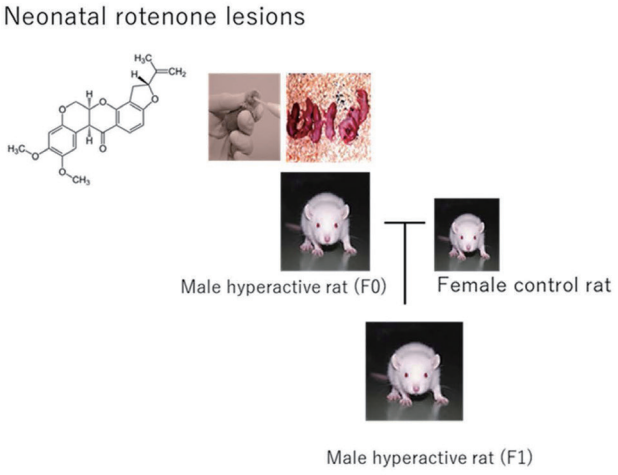

Fig. 1. Illustration of rat generation in this study. Rotenone (3 mg/kg; *top left*) was orally administered to male pups at 5 days of age (*top*), resulting in hyperactive rat (F0). Then, in adulthood, male hyperactive rat was mated with female control rat, resulting in hyperactive rat (F1; *bottom*). Note that the hyperactive rat (F0) never receives any rotenone.

paired infrared pyroelectric detectors that measure the radiated body heat of the animal. This system detects any object with a temperature at least 5°C higher than background within a cone-shaped area with a 6 m diameter and a 110°vertex. The sensor monitors motion in multiple zones of the cage through an array of Fresnel lenses placed above the cage and movement of the animal in the *X, Y,* and *Z* axis can be covered. A Supermex has the ability to analyze up to 64 channels with an optional instrument, an interface for data recording (DI-064W). Output of the sensor signals representing the magnitude of the rat's movement is transmitted by an interface device to a personal computer and is digitally converted and processed by the CompACT AMS software.

Spontaneous motor activity of the rats was individually measured in a home cage. We measured the activity counted by this system for 15 min intervals for a period of 22 hr. Food and water were fully available *ad libitum* from the beginning of measurement and rats were never disturbed in any way.

Statistics

Statistical analyses were carried out using the Microsoft Excel 365 software (Tokyo, Japan). Total activity in the nocturnal phase was analyzed by Student's t-test after ANOVA (analysis of variance).

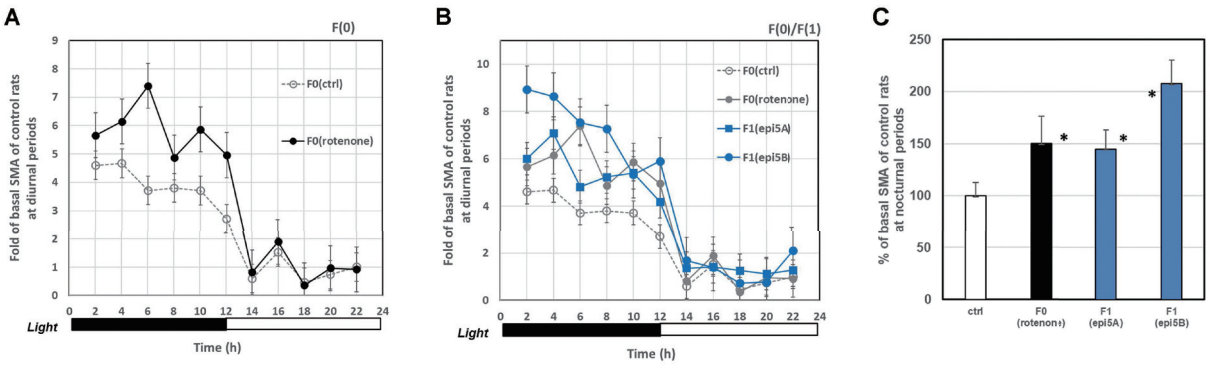

Fig. 2. Typical patterns of behavioral traits of each generation of neonatal rotenone lesion. *A;* Spontaneous motor activity (SMA) of neonatal rotenone (3 mg/kg)-treated rats (*filled circles*) was measured using the Supermex system at adulthood (F0). Measured activity during 2-hr interval is plotted. Control rats were given 30 µL of vehicle alone (*open circles*). Data were represented as a fold of the basal SMA of control rats (ctrl) at diurnal periods. *B;* Male hyperactive rats were mated with female control rats, resulting in hyperactive rats (F1), whose SMA was represented with that of F0 rats. There are two strains of F1 generation, designated as epi5A (*blue filled square*) and epi5B (*blue filled circles*). Data were represented as an fold of the basal SMA of control rats (*ctrl*) at diurnal periods. *C;* SMA during nighttime of all generations was integrated, as indicated. Data were represented as a percentage of those of control rats (*ctrl*), and indicated as mean ± s.e. *Significantly different from control rats ($p < 0.05$).

RESULTS AND DISCUSSION

To examine whether rat hyperactivity elicited by rotenone would be *de novo* inheritance or not, we first created hyperactive rats (F0) by neonatal rotenone lesion. Rotenone (3 mg/kg) was orally exposed to 5-day old male pups and their spontaneous motor activity was measured. Figure 2A shows that rotenone significantly increased motor activity through the nocturnal phase of the light-dark cycle.

We then examined the transgeneration of rat hyperactivity. Male control or male hyperactive rats (F0) were mated with female control rats from different litters, respectively. The offspring (F1) were obtained. Two F1 strains were established, designated as epi5A and epi5B, respectively. Their spontaneous motor activity was measured by Supermex system. Figure 2B shows that spontaneous motor activity of both F1 rats were significantly higher than that of control rats; they were comparable to that of F0. Figure 2C shows the total spontaneous motor activities in the dark periods (12 hr) of F0 and F1 rats. The extent of hyperactivity in offspring was larger than that of parents ($p < 0.05$). Spontaneous motor activity in F_0 treated rats was 1.3 times higher than that of control rats, while it was 1.5~2 times in F_1 offspring. Thus, this is not soft inheritance.

The vulnerability of the developing brain is dependent on, particularly the period of exposure to chemicals (Jacobson, 1991; Rice and Barone, 2000). In the 5-day old rats, developmental processes such as differentiation and synaptogenesis are incomplete (Vorhees, 1986; Rice and Barone, 2000).

In this study, I demonstrated that a single dose of rotenone (3 mg/kg) was orally administered to male pups at 5 days of age, being hyperactive at adulthood. The male hyperactive rats were then mated with female rat, resulting in the hyperactive generation (F1). The extent of hyperactivity was not decreased. This is strongly indicated the *de novo* inheritance of rotenone toxicity which was caused at F0 without soft matters.

Zhu *et al*. (2014) demonstrate that mouse hyperactivity, which was generated by mating of a female mouse preexposed to nicotine with a male mouse could be transmitted to offspring via the maternal line. The exposure of pregnant dams to nicotine was kept during pregnancy. It was mediated via a maternal line. However, *de novo* effect has not still been unknown.

Neonatal lesion by an epigenetic modifier, valproic acid could elicit rat hyperactivity (manuscript in preparation). Thus it is possible that rotenone toxicity seen in F0 might be mediated to F1 generation through epigenetic mechanism. This possibility is under investigation.

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Conflict of interest---- The authors declare that there is no conflict of interest.

REFERENCES

Anway, M.D., Cupp, A.S., Uzumcu, M. and Skinner, M.K. (2005): Epigenetic transgenerational actions of endocrine disruptors and male fertility. Science, **308**, 1466-1469.

Cho, S.-C., Bhang, S.-Y., Hong, Y.-C., Shin, M.S., Kim, B.N., Kim, J.-W., Yoo, H.-J., Cho, I.H. and Kim, H.-W. (2010): Relationship between environmental phthalate exposure and the intelligence of school-age children. Environ. Health Perspect., **118**, 1027-1032.

Chopra, V., Harley, K., Lahiff, M. and Eskenazi, B. (2014): Association between phthalates and attention deficit disorder and learning disability in U.S. children, 6-15 years. Environ. Res., **128**, 64-69.

Engel, S.M., Villanger, G.D., Nethery, R.C., Thomsen, C., Sakhi, A.K., Drover, S.S., Hoppin, J.A., Zeiner, P., Knudsen, G.P., Reichborn-Kjennerud, T., Herring, A.H. and Aase, H. (2018): Prenatal phthalates, maternal thyroid function, and risk of attention-deficit hyperactivity disorder in the Norwegian mother and child cohort. Environ. Health Perspect., **126**, 057004.

Franke, B., Faraone, S.V., Asherson, P., Buitelaar, J., Bau, C.H., Ramos-Quiroga, J.A., Mick, E., Grevet, E.H., Johansson, S., Haavik, J., Lesch, K.-P., Cormand, B. and Reif, A.; International Multicentre persistent ADHD CollaboraTion. (2012): The genetics of attention deficit/hyperactivity disorder in adults, a review. Mol. Psychiatry, **17**, 960-987.

Ghirardi, L., Pettersson, E., Taylor, M.J., Freitag, C.M., Franke, B., Asherson, P., Larsson, H. and Kuja-Halkola, R. (2019): Genetic and environmental contribution to the overlap between ADHD and ASD trait dimensions in young adults: a twin study. Psychol. Med., **49**, 1713-1721.

Goldman, L.S., Genel, M., Bezman, R.J. and Slanetz, P.J. (1998): Diagnosis and treatment of attention-deficit/hyperactivity disorder in children and adolescents. Council on Scientific Affairs, American Medical Association. JAMA, **279**, 1100-1107.

Gordon, S.M. and Mitchell, A.E. (2009) Attention deficit hyperactivity disorder (ADHD). Nova Biomedical Books, New York.

Gozal, D. and Molfese, D.L. (2005) Attention deficit hyperactivity disorders: From genes to patients, Human Press, New Jersey.

Harley, K.G., Aguilar Schall, R., Chevrier, J., Tyler, K., Aguirre, H., Bradman, A., Holland, N.T., Lustig, R.H., Calafat, A.M. and Eskenazi, B. (2013): Prenatal and postnatal bisphenol A exposure and body mass index in childhood in the CHAMACOS cohort. Environ. Health Perspect., **121**, 514-520.

Huang, H.-B., Chen, H.-Y., Su, P.-H., Huang, P.-C., Sun, C.-W., Wang, C.J., Chen, H.-Y., Hsiung, C.-A. and Wang, S.-L. (2015): Fetal and Childhood Exposure to Phthalate Diesters and Cognitive Function in Children Up to 12 Years of Age: Taiwanese Maternal and Infant Cohort Study. PLoS One, **10**, e0131910.

Ishido, M., Masuo, Y., Kunimoto, M., Oka, S. and Morita, M. (2004a): Bisphenol A causes hyperactivity in the rat concomitantly with impairment of tyrosine hydroxylase immunoreactivity. J. Neurosci. Res., **76**, 423-433.

Ishido, M., Masuo, Y., Sayato-Suzuki, J., Oka, S., Niki, E. and Morita, M. (2004b): Dicyclohexylphthalate causes hyperactivity in the rat concomitantly with impairment of tyrosine hydroxylase immunoreactivity. J. Neurochem., **91**, 69-76.

Ishido, M., Masuo, Y., Oka, S., Niki, E. and Morita, M. (2004c): *p*-Nitrotoluene causes hyperactivity in the rat. Neurosci. Lett., **366**, 1-5.

Ishido, M., Morita, M., Oka, S. and Masuo, Y. (2005): Alteration of gene expression of G protein-coupled receptors in endocrine disruptors-caused hyperactive rats. Regul. Pept., **126**, 145-153.

Ishido, M., Yonemoto, J. and Morita, M. (2007): Mesencephalic neurodegeneration in the orally administered bisphenol A-caused hyperactive rats. Toxicol. Lett., **173**, 66-72.

Ishido, M., Masuo, Y., Terasaki, M. and Morita, M. (2011): Rat hyperactivity by bisphenol A, but not by its derivatives, 3-hydroxybisphenol A or bisphenol A 3,4-quinone. Toxicol. Lett., **206**, 300-305.

Ishido, M. and Masuo, Y. (2014): Temporal effects of bisphenol A on dopaminergic neurons: an experiment on adult rats. Open Environ. Sci., **8**, 9-17.

Ishido, M., and Shimaya, E. (2016) Major histocompatibility complex expression in a rotenone model of Parkinson's disease in rats. **3**, 101-108.

Ishido, M. and Usu, R. (2017a): Orally administered *p*-nitrotoluene causes hyperactivity, concomitantly with gliosis and impairment of tyrosine hydroxylase immunoreactivity in the rat substantia nigra. Fundam. Toxicol. Sci., **4**, 151-158.

Ishido, M., Suzuki, J. and Masuo, Y. (2017b): Neonatal rotenone lesions cause onset of hyperactivity during juvenile and adulthood in the rat. Toxicol. Lett., **266**, 42-48.

Ishido, M. (2018): The temporal turning window for rat behavioral phenotypes by rotenone. Fundam. Toxicol. Sci., **5**, 195-202.

Jacobson, M. (1991): Developmental Neurobiology. 3rd ed. Plenum Press, New York.

Kim, B.N., Cho, S.C., Kim, Y., Shin, M.S., Yoo, H.J., Kim, J.W., Yang, Y.H., Kim, H.W., Bhang, S.Y. and Hong, Y.C. (2009): Phthalates exposure and attention-deficit/hyperactivity disorder in school-age children. Biol. Psychiatry, **66**, 958-963.

Ku, H.-Y., Tsai, T.-L., Wang, P.-L., Su, P.-H., Sun, C.-W., Wang, C.-J. and Wang, S.-L. (2020): Prenatal and childhood phthalate exposure and attention deficit hyperactivity disorder traits in child temperament: A 12-year follow-up birth cohort study. Sci. Total Environ., **699**, 134053.

LaHoste, G.J., Swanson, J.M., Wigal, S.B., Glabe, C., Wigal, T., King, N. and Kennedy, J.L. (1996): Dopamine D4 receptor gene polymorphism is associated with attention deficit hyperactivity disorder. Mol. Psychiatry, **1**, 121-124.

Martin, J., Cooper, M., Hamshere, M.L., Pocklington, A., Scherer, S.W., Kent, L., Gill, M., Owen, M.J., Williams, N., O'Donovan, M.C., Thapar, A. and Holmans, P. (2014): Biological overlap of attention-deficit/hyperactivity disorder and autism spectrum disorder: evidence from copy number variants. J. Am. Acad. Child Adolesc. Psychiatry, **53**, 761-70.e26.

Masuo, Y., Ishido, M., Morita, M. and Oka, S. (2004): Effects of neonatal treatment with 6-hydroxydopamine and endocrine disruptors on motor activity and gene expression in rats. Neural Plast., **11**, 59-76.

Park, S., Lee, J.-M., Kim, J.-W., Cheong, J.H., Yun, H.J., Hong, Y.-C., Kim, Y., Han, D.H., Yoo, H.J., Shin, M.-S., Cho, S.-C. and Kim, B.-N. (2015): Association between phthalates and externalizing behaviors and cortical thickness in children with attention deficit hyperactivity disorder. Psychol. Med., **45**, 1601-1612.

Philippat, C., Nakiwala, D., Calafat, A.M., Botton, J., De Agostini, M., Heude, B. and Slama, R.; EDEN Mother–Child Study Group. (2017): Prenatal exposure to nonpersistent endocrine disrupters and behavior in boys at 3 and 5 years. Environ. Health Perspect., **125**, 097014.

Polderman, T.J., Hoekstra, R.A., Posthuma, D. and Larsson, H. (2014): The co-occurrence of autistic and ADHD dimensions in adults: an etiological study in 17,770 twins. Transl. Psychiatry, **4**, e435.

Rappley, M.D. (2005): Clinical practice. Attention deficit-hyperactivity disorder. N. Engl. J. Med., **352**, 165-173.

Rice, D. and Barone, S. Jr. (2000): Critical periods of vulnerability for the developing nervous system: evidence from humans and animal models. Environ. Health Perspect., **108**, 511-533.

Shaywitz, B.A., Yager, R.D. and Klopper, J.H. (1976): Selective brain dopamine depletion in developing rats: an experimental model of minimal brain dysfunction. Science, **191**, 305-308.

Tewar, S., Auinger, P., Braun, J.M., Lanphear, B., Yolton, K., Epstein, J.N., Ehrlich, S. and Froehlich, T.E. (2016): Association of Bisphenol A exposure and Attention-Deficit/Hyperactivity Disorder in a national sample of U.S. children. Environ. Res., **150**, 112-118.

Yolton, K., Xu, Y., Strauss, D., Altaye, M., Calafat, A.M., Khoury, J., Yang, Y.H., Kim, H.-W., Yolton, K., Xu, Y., Strauss, D., Altaye, M., Calafat, A.M. and Khoury, J. (2011): Prenatal exposure to bisphenol A and phthalates and infant neurobehavior. Neurotoxicol. Teratol., **33**, 558-566.

Zhu, J., Lee, K.P., Spencer, T.J., Biederman, J. and Bhide, P.G. (2014): Transgenerational transmission of hyperactivity in a mouse model of ADHD. J. Neurosci., **34**, 2768-2773.